



UNIVERSITÀ
DEGLI STUDI
FIRENZE

FLORE

Repository istituzionale dell'Università degli Studi di Firenze

Senescence-associated beta-galactosidase is lysosomal beta-galactosidase.

Questa è la Versione finale referata (Post print/Accepted manuscript) della seguente pubblicazione:

Original Citation:

Senescence-associated beta-galactosidase is lysosomal beta-galactosidase / LEE BY; HAN JA; IM JS; A. MORRONE; JOHUNG K; GOODWIN EC; KLEIJER WJ; DIMAIO D; HWANG ES. - In: AGING CELL. - ISSN 1474-9718. - STAMPA. - 5 (2):(2006), pp. 187-195. [10.1111/j.1474-9726.2006.00199.x]

Availability:

This version is available at: 2158/216175 since: 2019-07-25T09:18:37Z

Published version:

DOI: 10.1111/j.1474-9726.2006.00199.x

Terms of use:

Open Access

La pubblicazione è resa disponibile sotto le norme e i termini della licenza di deposito, secondo quanto stabilito dalla Policy per l'accesso aperto dell'Università degli Studi di Firenze (<https://www.sba.unifi.it/upload/policy-oa-2016-1.pdf>)

Publisher copyright claim:

(Article begins on next page)

Senescence-associated β -galactosidase is lysosomal β -galactosidase

Bo Yun Lee,¹ Jung A. Han,¹ Jun Sub Im,¹ Amelia Morrone,² Kimberly Johung,³ Edward C. Goodwin,³ Wim J. Kleijer,⁴ Daniel DiMaio^{3,5} and Eun Seong Hwang^{1,3}

¹Department of Life Science, University of Seoul, Dongdaemungu, Jeonnonngdong, Seoul, Korea 130-743

²Metabolic and Muscular Disease Unit, Department of Pediatrics, University of Florence, Florence, Italy

³Department of Genetics, Yale University School of Medicine, New Haven, CT 06520-8005, USA

⁴Department of Clinical Genetics, Erasmus Medical Center, Rotterdam, The Netherlands

⁵Department of Therapeutic Radiology, Yale University School of Medicine, New Haven, CT 06520-8005, USA

Summary

Replicative senescence limits the proliferation of somatic cells passaged in culture and may reflect cellular aging *in vivo*. The most widely used biomarker for senescent and aging cells is senescence-associated β -galactosidase (SA- β -gal), which is defined as β -galactosidase activity detectable at pH 6.0 in senescent cells, but the origin of SA- β -gal and its cellular roles in senescence are not known. We demonstrate here that SA- β -gal activity is expressed from *GLB1*, the gene encoding lysosomal β -D-galactosidase, the activity of which is typically measured at acidic pH 4.5. Fibroblasts from patients with autosomal recessive G_{M1} -gangliosidosis, which have defective lysosomal β -galactosidase, did not express SA- β -gal at late passages even though they underwent replicative senescence. In addition, late passage normal fibroblasts expressing small-hairpin interfering RNA that depleted *GLB1* mRNA underwent senescence but failed to express SA- β -gal. *GLB1* mRNA depletion also prevented expression of SA- β -gal activity in HeLa cervical carcinoma cells induced to enter a senescent state by repression of their endogenous human papillomavirus E7 oncogene. SA- β -gal induction during senescence was due at least in part to increased expression of the lysosomal β -galactosidase protein.

These results also indicate that SA- β -gal is not required for senescence.

Key words: aging; cellular senescence; gene expression; *GLB1*, HeLa cells; lysosomes; molecular biology of aging; senescence.

Introduction

Normal somatic cells proliferate for a limited number of doublings in culture and then enter an irreversible growth-arrested stage called replicative senescence (Campisi, 2005). Key phenotypes of senescence include enlarged and flat cell morphology, β -galactosidase activity detectable at pH 6.0 [defined as senescence-associated β -galactosidase (SA- β -gal) activity] (Dimri *et al.*, 1995), and high-level autofluorescence due to lipofuscin accumulation (von Zglinicki *et al.*, 1995). Levels of p53, p21^{WAF1}, and p16^{INK4a} often gradually increase as fibroblasts approach senescence (Dimri *et al.*, 1996), and senescent cells produce high levels of reactive oxygen species (ROS) and contain elevated levels of oxidative DNA damage (Chen *et al.*, 1995; Song *et al.*, 2005). Permanent growth arrest that displays many of the features of replicative senescence can also be acutely induced by a variety of manipulations including expression of activated oncogenes (Serrano *et al.*, 1997; Zhu *et al.*, 1998), exposure to sublethal levels of DNA damaging agents or oxidative stress (Chen *et al.*, 1995; Shay & Roninson, 2004), and introduction or activation of tumor suppressor genes (Bringold & Serrano, 2000; Hwang, 2002). Cellular senescence is viewed as a model for certain aspects of cellular and organismal aging (Campisi, 2005), but the relationship between senescence and aging remains to be established. In addition, several recent studies strongly suggest that senescence plays a role in tumor suppression (Braig, 2005; Campisi, 2005; Collado, 2005; Michaloglou, 2005).

SA- β -gal is a β -galactosidase activity detectable at pH 6.0 in cultured cells undergoing replicative or induced senescence but absent from proliferating cells (Dimri *et al.*, 1995). SA- β -gal activity is typically measured by *in situ* staining using a chromogenic substrate such as X-gal. Since it was first reported, SA- β -gal activity has been the most extensively utilized biomarker for senescence because of the simplicity of the assay method and its apparent specificity for senescent cells. It is not clear whether a process analogous to senescence occurs during aging in animals. SA- β -gal activity has been detected in organs of old individuals and animals, suggesting that cellular senescence is a feature of organismal aging and that senescent cells accumulate with age in tissue (e.g. Dimri *et al.*, 1995; Mishima *et al.*, 1999; Pendergrass *et al.*, 1999; Sigal *et al.*, 1999; Melk *et al.*,

Correspondence

Daniel DiMaio, Department of Genetics, Yale University School of Medicine, PO Box 208005, New Haven, CT 06520-8005, USA. Tel.: 203-785-2684; fax: 203-785-6765; e-mail: daniel.dimaio@yale.edu,

Eun Seong Hwang, Department of Life Science, University of Seoul, Dongdaemungu, Jeonnonngdong, Seoul, Korea 130-743.

Tel.: 82-2-2210-2608; fax: 82-2-2210-2888

e-mail: eshwang@uos.ac.kr

Accepted for publication 21 December 2005

2003). In some cases, the identification of cells as senescent rests solely on the detection of SA- β -gal activity. However, despite the widespread reliance on SA- β -gal as a marker of senescence, the origin of SA- β -gal activity has not been conclusively determined, and its role in senescence, if any, is unknown. In fact, there are a number of reports that β -galactosidase activity at pH 6.0 can be detected in cells in various nonsenescent states, such as extended incubation at high density, and there are conflicting reports regarding the presence of SA- β -gal activity in aged tissues (e.g. Yegorov *et al.*, 1998; Krishna *et al.*, 1999; Kurz *et al.*, 2000; Severino *et al.*, 2000; Untergasser *et al.*, 2003). Thus, the suitability of SA- β -gal as a marker of senescence has been challenged (Krishna *et al.*, 1999; Severino *et al.*, 2000; Coates, 2002; Cristofalo, 2005; Yang & Hu, 2005).

It is essential to establish the origin of SA- β -gal activity to determine the basis for its induction during senescence and its suitability as a marker that unambiguously identifies senescent cells. One candidate protein that may underlie SA- β -gal activity is a well-characterized β -D-galactosidase (EC 3.3.1.23) that is localized to lysosomes of mammalian cells. Consistent with localization in this acidic organelle, lysosomal β -galactosidase displays maximal activity between pH 4.0 and 4.5 but markedly lower activity at pH 6.0 (Zhang *et al.*, 1994). Indeed, β -galactosidase activity is not detectable in proliferating cells by *in situ* staining with X-gal at pH 6.0, the conditions used to detect SA- β -gal activity, even though lysosomal β -galactosidase activity is readily detectable in these cells at acidic pH. Nevertheless, based on indirect physiological experiments, it has been proposed that increased lysosomal- β -galactosidase activity in senescent cells accounts for SA- β -gal activity (Kurz *et al.*, 2000). Specifically, the levels of total cellular β -galactosidase activity is higher in late-passage compared to early-passage cells at pH 4.5 as well as at pH 6.0, and maximal β -galactosidase activity is measurable at low pH in both early- and late-passage cells (Ferland *et al.*, 1990; Kurz *et al.*, 2000; Gerland *et al.*, 2003; Gary & Kindell, 2005; Yang & Hu, 2005). Furthermore, the number and size of lysosomes increase in cells at late passage (Robbins *et al.*, 1970; Brunk *et al.*, 1973). These results suggest that lysosomal β -galactosidase activity increases in senescent cells due to increased lysosome content, surpassing a threshold level so that it is detectable at the suboptimal pH 6.0 (Kurz *et al.*, 2000; Gary & Kindell, 2005). However, no experiments have been reported that directly test the hypothesis that SA- β -gal activity is due to increased amounts or activity of the lysosomal- β -galactosidase protein in senescent cells, and not due to increased activity of another enzyme capable of catalyzing the hydrolysis of terminal β -D-galactose residues from β -galactosides. In this paper, we provide compelling genetic evidence that SA- β -gal activity is in fact encoded by the lysosomal- β -galactosidase gene (designated *GLB1*) and that levels of lysosomal- β -galactosidase protein increase during senescence. In addition, we demonstrate that SA- β -gal activity is not required for senescence.

Results

Low lysosomal β -galactosidase activity in G_{M1} -gangliosidosis fibroblasts with inactivating mutations in the *GLB1* gene

SKa and JNa primary fibroblasts were established from skin biopsies of G_{M1} -gangliosidosis patients. Patients with this autosomal recessive disease lack lysosomal β -galactosidase (EC 3.2.1.23) activity (Dacremont & Kint, 1968; Norden & O'Brien, 1975). DNA sequencing of the lysosomal β -galactosidase gene (designated *GLB1*) revealed the existence of a homozygous arg201cys mutation in SKa cells and a homozygous arg59his mutation in JNa cells. Both mutations are known to result in the loss of lysosomal β -galactosidase activity and G_{M1} -gangliosidosis (Silva *et al.*, 1993; Oshima *et al.*, 1994; Caciotti *et al.*, 2005). As expected, extracts of cultured SKa and JNa fibroblasts contained low levels of β -galactosidase activity measured at pH 4.5 as compared to the normal fibroblasts (Fig. 1A). Furthermore, when SKa and JNa fibroblasts were incubated in the presence of X-gal at pH 4.5, no cells stained positively, whereas virtually all of the normal fibroblasts stained intensely (data not shown).

Replicative senescence in G_{M1} -gangliosidosis fibroblasts

Proliferation of the SKa and JNa fibroblasts reached a Hayflick limit (Hayflick & Moorhead, 1961) after comparable numbers of population doublings (PD) in cell culture. As judged by the lack of a significant increase in cell number during a 20-day culture period, the control normal fibroblasts proliferated to PD75, whereas the SKa and JNa fibroblasts proliferated to approximately PD59 and PD58, respectively (data not shown). (The PD number for the G_{M1} -gangliosidosis cells is an underestimate because PD numbers at some early passages were not recorded). The acquisition of a senescent state in these late-passage cells was confirmed by several criteria in addition to growth retardation. In senescent normal fibroblasts, the levels of p53 and p21^{WAF1} or p16^{INK4a} are high, the p105^{Rb} retinoblastoma protein is primarily hypophosphorylated, and cellular levels of autofluorescence and ROS increase. At late passages, the normal and G_{M1} -gangliosidosis cell strains displayed elevated levels of p53 and p21^{WAF1} as well as hypophosphorylated Rb (Fig. 1B shows the status of p21^{WAF1} as a representative example). All three cell strains also displayed increased cell size, flattened morphology, autofluorescence, and ROS at the late passages but not at the early passages (Fig. 1C and data not shown). Similar changes in all three cell strains at early passage were also elicited by adriamycin treatment, which induces a state of senescence in normal fibroblasts (B.Y.L. and E.S.H., unpublished result). Taken together, these results indicate that senescence could be imposed on the G_{M1} -gangliosidosis fibroblasts either by prolonged replication in culture or by chemical stress.

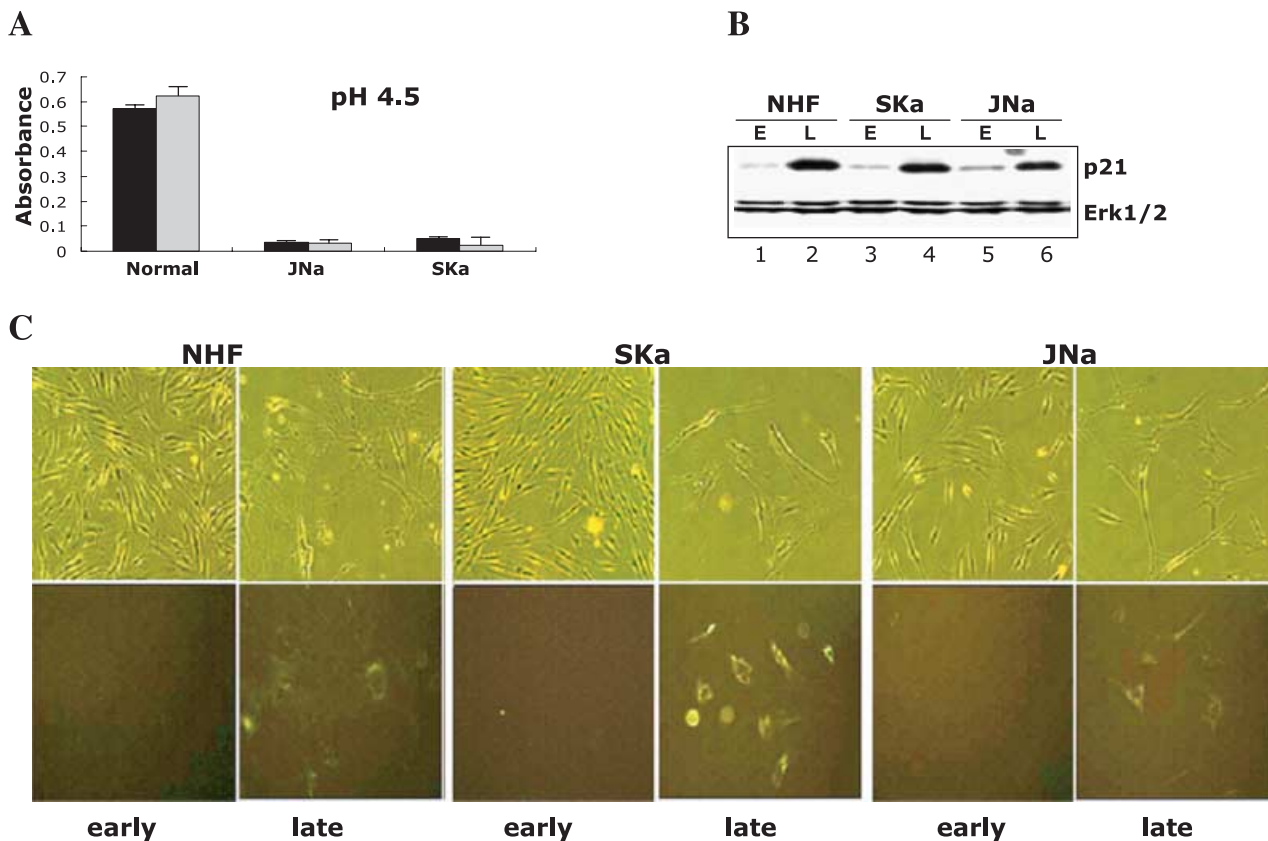


Fig. 1 Characterization of G_{M1} -gangliosidosis fibroblasts. (A) 10^5 early-passage normal, JNa, and SKa fibroblasts were lysed in 100 μ L of 0.1 M citrate buffer (pH 4.5). Equal volumes of extract were assayed in solution for acidic β -galactosidase activity (pH 4.5) using ONPG as substrate. Each bar shows the average of three assays with the cells prepared at either PD23 (black bars) or PD26 (gray bars). (B) p21^{WAF1} levels in normal, SKa, and JNa cells. Extracts (20 μ g protein) from cells collected either at early passage (E) (PD18 for the normal and SKa cells, and PD24 for JNa) or late passage (L) (PD74, PD58, and PD56 for the normal, SKa, and JNa cells, respectively) were subjected to Western blotting for p21^{WAF1} and Erk-1/2. (C) Early- and late-passage fibroblasts were mounted and examined by fluorescence (bottom panels) and phase contrast microscopy (top panels). NHF, normal human fibroblasts.

Absence of SA- β -gal activity in G_{M1} -gangliosidosis fibroblasts

The senescent SKa and JNa fibroblasts were analyzed for SA- β -gal activity *in situ* by incubation with X-gal at pH 6.0. Although the great majority of normal cells stained positively for SA- β -gal activity at late passage, none of the late-passage SKa or JNa cells stained (Fig. 2A). Enzymatic activity assays in cell extracts also demonstrated very low β -galactosidase activity at pH 6.0 in senescent G_{M1} -gangliosidosis fibroblasts, whereas the late-passage normal cells displayed high level SA- β -gal activity (approximately sixfold higher than the level in early-passage cells) (Fig. 2B). Similarly, adriamycin-treated normal fibroblasts, but not G_{M1} -gangliosidosis fibroblasts, displayed *in situ* SA- β -gal activity (data not shown). Therefore, SA- β -gal activity is absent or markedly reduced in G_{M1} -gangliosidosis fibroblasts, which are devoid of active lysosomal β -galactosidase due to inactivating mutations in the *GLB1* gene, strongly suggesting that lysosomal β -galactosidase is the origin of SA β -gal activity.

Decreased SA- β -gal activity in late-passage fibroblasts expressing *GLB1* interfering RNA

We used an independent approach to confirm the origin of SA- β -gal activity. Pre-senescent (PD62) normal fibroblasts were infected in parallel with retroviruses that expressed two different small hairpin interfering RNAs (shRNA) targeting *GLB1* mRNA, along with the gene for puromycin-resistance, or with a control retrovirus. Following puromycin selection, pooled cell lines were established and analyzed. Real-time RT-PCR demonstrated that the level of *GLB1* mRNA decreased by 7.3- and 5.6-fold, respectively, in two independent lines that express shRNA targeting either of two different sites in the *GLB1* mRNA (data not shown). Repression of lysosomal β -galactosidase expression was confirmed by Western blotting (see Fig. 4, lane 5). The level of *GLB1* mRNA was not affected in control cells that express *Photinus* luciferase shRNA (shLUC). In both *GLB1*-knockdown fibroblast lines, β -galactosidase activity measured at pH 4.5 in solution was reduced to less than 20% of that in the cells expressing luciferase shRNA or the parent cells at similar passage

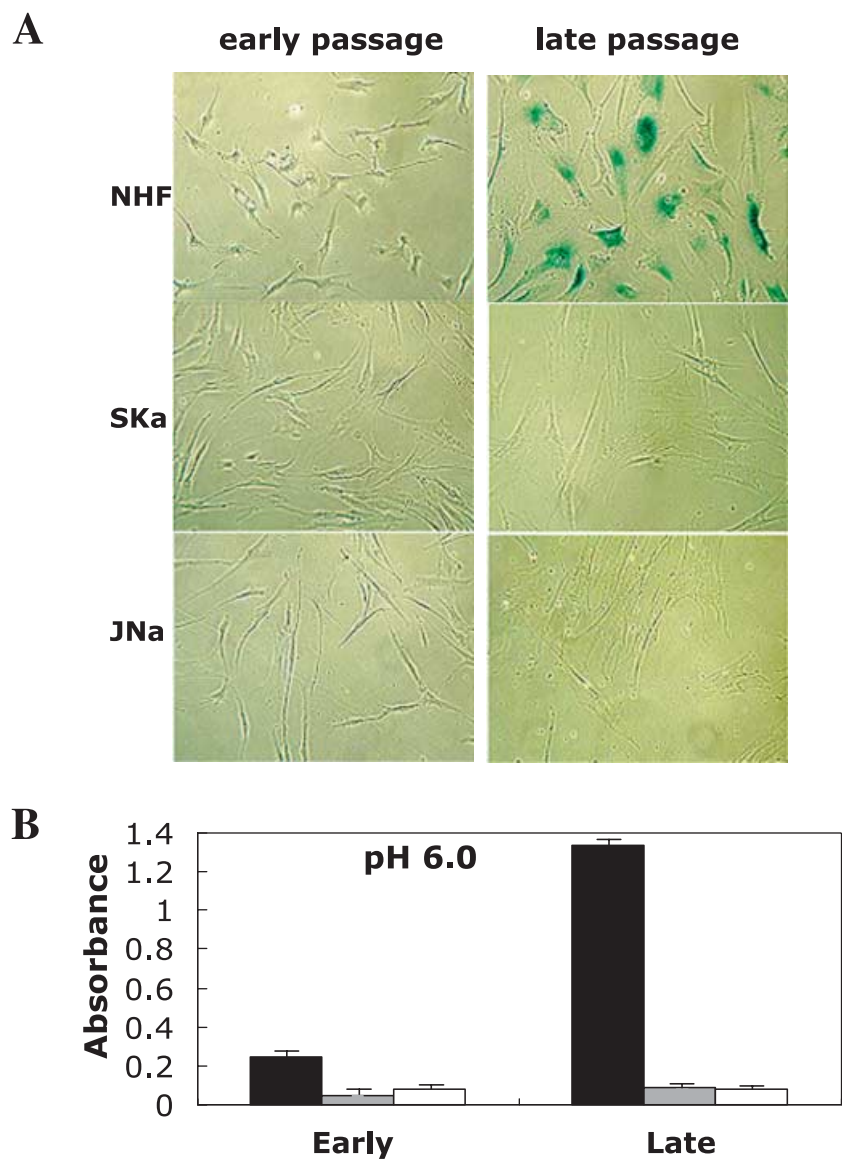


Fig. 2 Low SA-β-gal activity in G_{M1}-gangliosidosis fibroblasts. (A) Fibroblasts at either early or late passage (at PD indicated in legend of Fig. 1B) were subjected to *in situ* SA-β-gal staining at pH 6, and examined by bright field microscopy. (B) 10⁵ normal (black bar), SKa (grey bar), and JNa (white bar) cells were collected at either early or late passage as in panel A and lysed in 100 μL of 0.1 M phosphate buffer (pH 6.0), and equal volumes were assayed in solution for β-galactosidase activity at pH 6.0.

Table 1 Percentage of cells positive for SA-β-gal*

Vector	NHF (PD73)	HeLa	
		(-E2)	(+E2)
Empty	54.0 ± 8.4	2.5 ± 1.0	80.7 ± 5.9
ShGLB#2	5.1 ± 1.4	6.5 ± 1.6	4.6 ± 1.2
ShLUC	49.6 ± 11.9	n.d.	n.d.

*Two or more different fields with 50 or more cells were photographed, and number of cells stained blue with X-gal at pH 6.0 was divided by the total cell number.
n.d., not determined.

(Fig. 3A), and fewer than 10% of *GLB1*-knockdown fibroblasts positively stained *in situ* for acidic β-galactosidase at pH 4.5 (data not shown). Cells expressing *GLB1* shRNA and control cells were passaged until they ceased proliferation and assumed a

senescent morphology (at estimated PD73), at which time they were analyzed for *in situ* SA-β-gal activity at pH 6.0 (Fig. 3B and Table 1). As expected, many cells in the population of both the parental and the control shLUC lines positively stained. Strikingly, only rare *GLB1*-knockdown cells stained for SA-β-gal activity at pH 6.0, and the intensity of staining was in general lower in the rare positive cells (e.g. shGLB#2, Fig. 3B). In addition, β-galactosidase activity measured in solution at pH 6.0 was approximately 16.7% of the activity of the parent normal human fibroblasts (data not shown). Thus, *GLB1* mRNA suppression markedly inhibited SA-β-gal activity in senescent fibroblasts.

Decreased SA-β-gal activity in senescent HeLa cells expressing *GLB1* interfering RNA

HeLa cervical carcinoma cells can be induced to enter a senescent state by transduction of the bovine papillomavirus E2 gene,

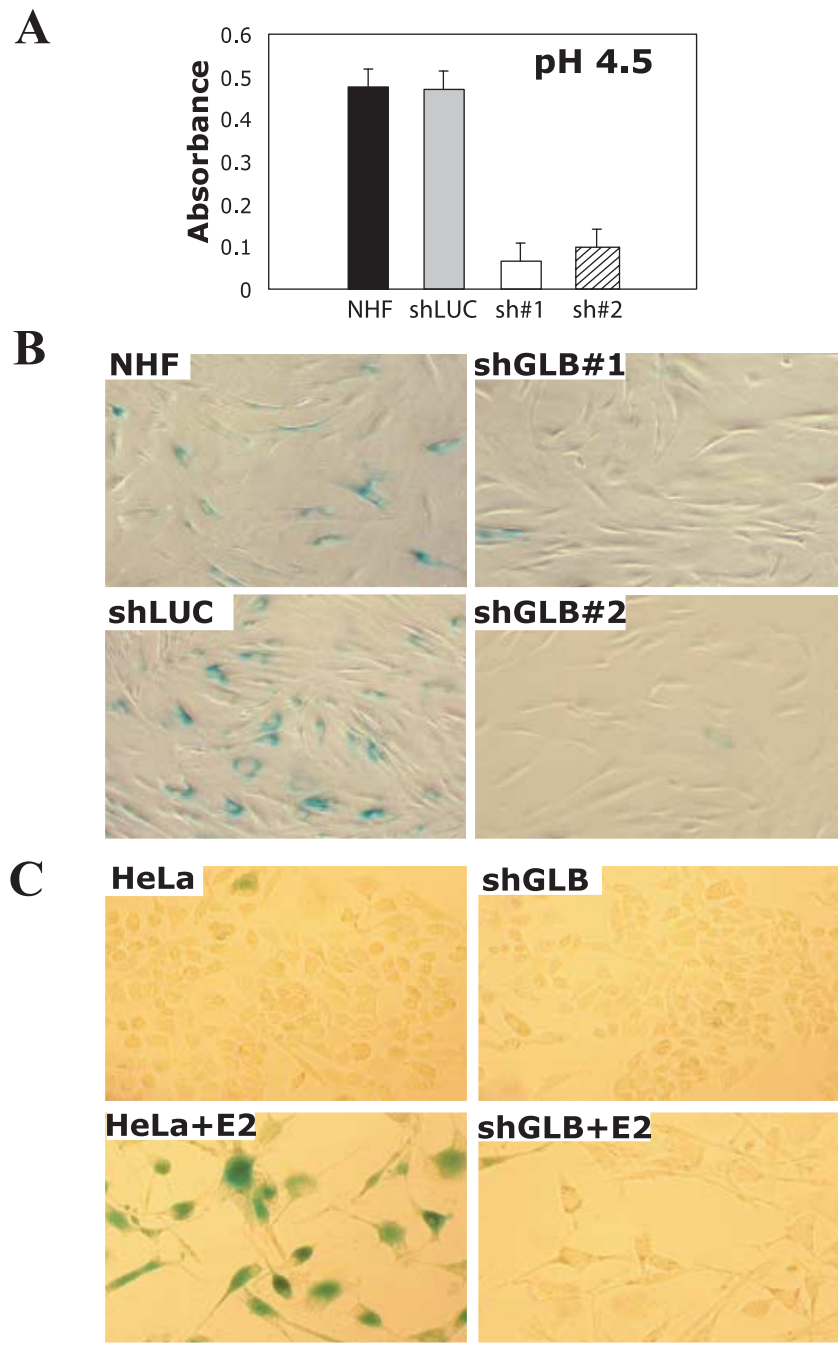


Fig. 3 Analysis of normal fibroblasts and HeLa cells expressing *GLB1* shRNA. (A) and (B) Normal fibroblasts were infected with retroviruses expressing shRNA targeting the *GLB1* mRNA (shGLB#1 and shGLB#2) or with a control retrovirus (shLUC), as indicated. Pooled puromycin-resistant cells were subjected to an assay for β -galactosidase in solution at pH 4.5 (A) or to *in situ* staining for β -galactosidase at pH 6.0 (B). Normal late-passage fibroblasts were stained as controls. (C) HeLa or HeLa-shGLB#2 cells were either mock infected or infected with the E2 virus, incubated for 10 days, and subjected to *in situ* staining for β -galactosidase at pH 6.0.

which suppresses HPV18 oncogene expression and activates tumor suppressor pathways in these cells (Hwang *et al.*, 1993; Goodwin *et al.*, 2000; Wells *et al.*, 2000). After expression of the E2 protein, cells rapidly and irreversibly arrest in G1 phase, and nearly 100% of them express a senescent phenotype, including elevated SA- β -gal activity. HeLa cells were modified to express decreased amounts of *GLB1* mRNA by infecting with the shGLB-retroviruses described above. In cells expressing shGLB#2, *GLB1* mRNA level was 8.1-fold lower than that in the mock-treated cells as determined by real-time RT-PCR (data not

shown). The *GLB1*-knockdown and control HeLa cells were infected with a virus that expressed the E2 protein, incubated for 10 days, and assayed for the SA- β -gal activity *in situ*. The parental HeLa cells infected with the E2 virus expressed the expected senescent phenotype including growth arrest, increased cell size and flattening, elevated autofluorescence and high level SA- β -gal activity (Fig. 3C, left panels, and data not shown). The *GLB1*-knockdown HeLa cells treated in parallel with the E2 gene also underwent growth arrest and displayed increased cell size and flattening and elevated autofluorescence

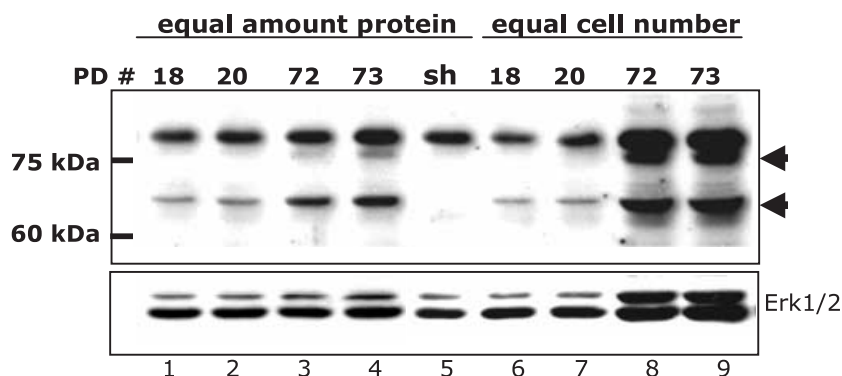


Fig. 4 Biochemical analysis of lysosomal β -galactosidase expression. 4.5×10^5 normal human fibroblasts at two early passages (PD18 and 20) and two late passages (PD72 and 73) as well as NHF-shGLB#2 at PD73 (lane 5) were lysed in 80 μ L of RIPA buffer. Either equal mass of extracted protein (10 μ g) (lanes 1–5) or equal volumes of extract (4×10^4 cell equivalent) (lanes 6–9) were subjected to Western blotting for human lysosomal β -galactosidase (top panel) or Erk-1/2 protein (bottom panel). The arrow indicates the lysosomal β -galactosidase band at approximately 64 kDa.

(Fig. 3C, right panels, and data not shown). Strikingly, on average < 5% of infected HeLa cells expressing the shGLB#2 RNA stained positively for SA- β -gal activity *in situ*, whereas 80% of the parental cells were positive (Fig. 3C and Table 1). Similar results were obtained in an independent population of HeLa cells generated by using the shGLB#1 virus (data not shown). β -Galactosidase activity measured in solution at pH 6.0 showed a progressive increase with increasing time after E2-infection of the parental cells, with little activity present in the *GLB1*-knock-down cells (data not shown). Thus, SA- β -gal activity in cancer cells induced to enter senescence also requires expression of the *GLB1* gene. Taken together, these results indicate that SA- β -gal activity originates from the *GLB1* gene in both replicative and induced senescence and that this activity is not required for senescence.

Increased lysosomal β -galactosidase protein in fibroblasts undergoing senescence

Although a protein that cross-reacts with an antibody raised to *Escherichia coli* β -galactosidase undergoes a progressive increase in senescing cells (Kurz *et al.*, 2000), the levels of lysosomal β -galactosidase protein have not been directly measured during senescence. To determine whether increased levels of lysosomal β -galactosidase protein accompanied the appearance of SA- β -gal activity in senescent cells, we conducted Western blotting with an antibody specific for human lysosomal β -galactosidase (Zhang *et al.*, 1994). Two samples each of early-passage and late-passage normal fibroblasts were prepared and normalized for either total protein content (Fig. 4, lanes 1–5) or cell number (Fig. 4, lanes 6–9). The 85 kDa precursor and the ~64 kDa mature form of the lysosomal β -galactosidase are readily detectable in early- and late-passage fibroblasts, except in late-passage cells expressing the *GLB1* shRNA (lane 5), as expected. Strikingly, there was an approximately sixfold increase in the amount of the 65 kDa protein band on a per cell basis in late-passage compared to early-passage cells (compare lanes 6 and 7 to 8 and 9). Correspondingly, β -galactosidase activity in solution increased approximately six- to sevenfold per cell in late-passage fibroblasts undergoing senescence compared to early-passage cells when the assay was conducted at either pH 4.5 or 6.0, although the absolute level of activity was higher

at pH 4.5 in both early- and late-passage cells (data not shown). Thus, increased SA- β -gal activity in senescent fibroblasts is due at least in part to increased levels of lysosomal β -galactosidase. Senescent cells are larger than early-passage cells, and approximately 2.5-fold more protein or RNA is isolated from equal numbers of late-passage compared to early-passage cells. Thus, if lysosomal β -galactosidase protein (Fig. 4, lanes 1–4) or SA- β -gal activity (data not shown) is expressed after normalization for total protein content (rather than on a per-cell basis), the increase in the senescent cells is approximately two- to threefold.

Levels of *GLB1* RNA were measured by quantitative real-time RT-PCR. This analysis revealed a 1.6-fold increase in the *GLB1* mRNA in late-passage fibroblasts normalized to the levels of γ -actin mRNA (average of independent measurements of three different sets of RNA samples, data not shown). On a per-cell basis, this corresponds to an approximately fourfold increase in *GLB1* mRNA in senescent cells.

Discussion

Since described in 1995 (Dimri *et al.*, 1995), *in situ* SA- β -gal activity has been widely used as a biomarker of senescence. Although several lines of indirect evidence suggested that SA- β -gal may be due to increased lysosomal β -galactosidase in senescent cells, the origin of SA- β -gal activity and the basis for induction has not been unequivocally established. In the present study, we provide two compelling pieces of genetic evidence that lysosomal β -galactosidase is the origin of SA- β -gal activity. First, senescent human G_{M1} -gangliosidosis fibroblasts lacking lysosomal β -galactosidase due to homozygous inactivating mutations in the *GLB1* gene fail to express SA- β -gal activity; second, SA- β -gal activity is greatly reduced in senescent cells in which the concentration of *GLB1* mRNA is reduced by RNA interference. Our results, together with published results from other laboratories (Kurz *et al.*, 2000; Gerland *et al.*, 2003; Gary & Kindell, 2005; Yang & Hu, 2005), demonstrate that lysosomal β -galactosidase activity increases enough in senescent cells to be detected at suboptimal pH 6.0, constituting SA- β -Gal activity. This increase appears to be due at least in part to the accumulation of increased levels of *GLB1* mRNA and protein. However, the extent of the senescence-induced increase in lysosomal β -galactosidase measured by Western blotting or

soluble enzymatic activity appears much less than the induction of SA- β -gal observed by *in situ* staining with X-gal. Thus, in addition to increased amounts of lysosomal β -galactosidase in senescent cells, functional differences in senescent lysosomes or other factors may contribute to the very high levels of β -galactosidase activity as assessed by *in situ* staining at pH 6.

Our finding that lysosomal β -galactosidase is the source of SA- β -gal activity in senescent cells indicates that SA- β -gal is not a specific marker of senescence *per se*, but rather a surrogate marker for increased lysosome number or activity, which has long been associated with replicative senescence and organismal aging (Robbins *et al.*, 1970; Brunk *et al.*, 1973; Turk & Milo, 1974; Cristofalo & Kabakjian, 1975; Knook *et al.*, 1975; Bosmann *et al.*, 1976; Sanchez-Martin & Cabezas, 1997; Gerland *et al.*, 2003). This is consistent with increased SA- β -gal activity in a number of nonsenescent situations and suggests that other conditions characterized by increased lysosomal content will also display elevated β -galactosidase activity at pH 6.0, and, conversely, that other lysosomal proteins are likely to increase during senescence and may also serve as senescence markers. Hence, SA- β -gal activity cannot stand alone to define a senescent state. Rather, senescence is best defined as the appearance of a constellation of features, only one of which is β -galactosidase activity at pH 6. The identification of *GLB1* as the gene encoding SA- β -gal will allow a systematic evaluation of the factors that control its expression in a variety of growth states, and thereby improve its use as a marker of senescence.

Our results highlight two additional points regarding senescence. First, *GLB1* is essential for expression of SA- β -gal in both replicative senescence and induced senescence, indicating at least partial overlap in the programs that lead to these senescence states. Second, the G_{M1} -gangliosidosis cells undergo senescence in response to serial passage or adriamycin treatment, and *GLB1* mRNA knockdown did not interfere with senescence, even though it caused a substantial reduction in SA- β -gal activity. Thus, SA- β -gal activity is not required for senescence, and the increase in β -galactosidase activity in senescent cells is an outcome rather than a cause of senescence.

Experimental procedures

Cell culture

Normal primary fibroblasts were isolated from healthy newborn foreskins and provided by Dr Sang Chul Park (Seoul National University, Korea) or the Yale Skin Diseases Research Center. G_{M1} -gangliosidosis fibroblasts (SKa and JNa cells) were isolated from skin biopsies of two patients clinically diagnosed as affected by G_{M1} -gangliosidosis (patients 89RD0051 and 90RD0450, respectively). The DNA sequence of the *GLB1* gene from these patients was determined as described (Caciotti *et al.*, 2005). Fibroblast cultures were passaged in low glucose (5.5 mM) DMEM containing 10% FBS at 1 : 4 ratio until the Hayflick limit (Hayflick & Moorhead, 1961) was reached. The number of population doublings (PD) was calculated using the

equation, $PD = \log_2 F/I$, where F and I are the numbers of cells at the end and those seeded at the beginning of one passage, respectively. To induce a state of senescence in early-passage fibroblasts (PD18 for normal and SKa cells, and PD24 for JNa cells), cells were treated with 0.5 μ M adriamycin for 4 h and then incubated in its absence for 5 days.

Senescence induction in HeLa cells

Senescence was induced in HeLa/E6.5K cells (DeFilippis *et al.*, 2003; referred to here as HeLa cells) as previously described by infection at multiplicity of infection of 20 with an SV40-recombinant virus (Pava1, designated here the E2 virus) expressing the bovine papillomavirus E2 protein (Hwang *et al.*, 1993; Goodwin *et al.*, 2000). This treatment represses the human papillomavirus (HPV) type 18 E7 gene, resulting in the acquisition of a number of markers of senescence, including growth arrest and an acute increase in SA- β -gal activity (Goodwin *et al.*, 2000; Kang *et al.*, 2004).

Detection of autofluorescence

Cells grown on glass cover slips were washed with phosphate-buffered saline (PBS), mounted in Gel/Mount (Biomedex, Foster City, CA, USA), and observed by fluorescence microscopy with an excitation filter (450–480 nm) and emission filter (520 nm) at $\times 200$ magnification. Autofluorescence in HeLa cells was measured by fluorescence activated cell sorting as described (Goodwin *et al.*, 2000).

Western blotting analysis

Cells were lysed in RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS] supplemented with protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO, USA), NaF, and NaVO_4 . Twenty μ g protein were subjected to gel electrophoresis as described (DeFilippis *et al.*, 2003) and analyzed by Western blotting using anti-p53 (DO-1; Calbiochem, San Diego, CA, USA), anti-p21^{WAF1} (C-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Rb (BD Biosciences, Franklin Lakes, NJ, USA), or anti-ERK 1/2 antibody. To detect lysosomal β -galactosidase protein, lysate from an equal number of cells or 20 μ g of extracted protein were electrophoresed and probed with a human lysosomal β -galactosidase specific polyclonal antiplacental β -galactosidase IgG, kindly provided by John Callahan (Hospital for Sick Children, Toronto, ON, Canada) (Zhang *et al.*, 1994). Protein bands were quantitated by using the ImageQuant 5.2 program (Amersham Biosciences, Piscataway, NJ, USA).

In situ staining for β -galactosidase activity

Cultured cells were washed in PBS (pH 7.4), fixed with 3.7% formaldehyde, and incubated overnight at 37 °C in freshly prepared staining buffer [1 mg mL⁻¹ X-gal (5-bromo-4-chloro-3-indolyl

β -D-galactoside), 5 mM $K_3Fe[CN]_6$, 5 mM $K_4Fe[CN]_6$, and 2 mM $MgCl_2$ in PBS, pH 6.0, or in citrate-buffered saline, pH 4.5]. At the end of the incubation, cells were washed with H_2O and examined at $\times 200$ magnification.

Soluble β -galactosidase assay

Equal numbers of cells were collected, washed and resuspended in either 0.1 M citrate (pH 4.5) or phosphate buffer (pH 6.0). Cells were lysed by freeze/thaw. The lysates were centrifuged at 12 000 g for 7 min. The supernatants were mixed with 2-nitrophenyl- β -D-galactopyranoside (ONPG) (Sigma) ($2.2 \mu g \mu L^{-1}$), 1 mM $MgCl_2$ in either the citrate or phosphate buffer. After incubation at 37 °C for 12 h, two volumes of 1 M sodium carbonate were added and absorbance at 420 nm was measured.

Knockdown of lysosomal β -galactosidase RNA

To generate retroviral vectors that express small hairpin RNA (shRNA) uniquely targeting the human *GLB1* mRNA (M34423), oligodeoxynucleotides containing 21-base target sequences (sh#1: 5'-ATGTAGCGAAATGGCTGGCCA-3'; sh#2: 5'-AAGT-GTTGTCCGGTACAGCAC-3') were annealed and ligated into pSIREN-RetroQ (BD Biosciences, San Jose, CA, USA) 3' to the human U6 promoter. The presence of the correct inserts was confirmed by sequencing. A control vector that expresses shRNA targeting *Photinus* luciferase (RV-shLUC) (BD Biosciences) was constructed in parallel. Retroviruses were packaged in 293T cells and concentrated. Pre-senescent normal fibroblasts (PD62) and HeLa cells were infected with these viruses individually and selected for 3–14 days for resistance to $0.4 \mu g mL^{-1}$ puromycin and then pooled. The resistant pools were maintained by continuous passage in $0.2 \mu g mL^{-1}$ puromycin. RNA was isolated using RNeasy kit (QIAGEN Sciences, Germantown, MD, USA) and converted to cDNA using iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) by following manufacturer's protocols. The protocol used to quantitate *GLB1* and γ -actin mRNA by single-color quantitative Real-Time PCR (Bio-Rad) is available from the authors upon request.

Acknowledgments

We thank John Callahan for the gift of antilyosomal β -galactosidase antibody, Kristin Yates for valuable discussions, and Jan Zulkowski for assistance with manuscript preparation. K.J. was supported in part by an MSTP grant to Yale University. This work was supported by a grant to E.S.H. from Korea Science and Engineering Foundation (KOSEF) through the Center for Aging and Apoptosis Research (R11-2002-097-07002-0) and a grant to D.D. from the National Cancer Institute (CA16038).

References

Bosmann HB, Gutheil RL, Case KR (1976) Loss of critical neutral protease in ageing WI-38 cells. *Nature* **261**, 499–501.

- Braig M, Lee S, Loddenkemper C, Rudolph C, Peters AHFM, Schlegelberger B, Stein H, Dörken B, Jenuwein T, Schmitt CA (2005) Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature* **436**, 660–665.
- Bringold F, Serrano M (2000) Tumor suppressors and oncogenes in cellular senescence. *Exp. Gerontol.* **35**, 317–329.
- Brunk UT, Ericsson JLE, Ponten J, Westermark B (1973) Residual bodies and 'aging' in cultured human glia cells. *Exp. Cell Res.* **79**, 1–14.
- Caciotti A, Donati MA, Boneh A, d'Azzo A, Federico A, Parini R, Antuzzi D, Bardelli T, Nosi D, Kimonis V, Zammarchi E, Morrone A (2005) Role of β -galactosidase and elastin binding protein in lysosomal and non-lysosomal complexes of patients with G_{M1} -gangliosidosis. *Hum. Mutat.* **25**, 285–292.
- Campisi J (2005) Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. *Cell* **120**, 513–522.
- Chen Q, Fischer A, Reagan JD, Yan LJ, Ames BN (1995) Oxidative DNA damage and senescence of human diploid fibroblast cells. *Proc. Natl Acad. Sci. USA* **92**, 4337–4341.
- Coates PJ (2002) Markers of senescence? *J. Pathol.* **196**, 371–373.
- Collado M, Gil J, Efeyan A, Guerra C, Schuhmacher AJ, Barradas M, Benguria A, Zaballos A, Flores JM, Barbacid M, Beach D, Serrano M (2005) Tumour biology: senescence in premalignant tumours. *Nature* **436**, 642.
- Cristofalo VJ (2005) SA β Gal staining: biomarker or delusion. *Exp. Gerontol.* **40**, 836–838.
- Cristofalo VJ, Kabakjian J (1975) Lysosomal enzymes and aging *in vitro*: subcellular enzyme distribution and effect of hydrocortisone on cell life-span. *Mech. Ageing Dev.* **4**, 19–28.
- Dacremont G, Kint JA (1968) G_{M1} -ganglioside accumulation and β -galactosidase deficiency in a case of G_{M1} -gangliosidosis (Landing disease). *Clin. Chim. Acta* **21**, 421–425.
- DeFilippis R, Goodwin EC, Wu L, DiMaio D (2003) Endogenous human papillomavirus E6 and E7 proteins differentially regulate proliferation, senescence, and apoptosis in HeLa cervical carcinoma cells. *J. Virol.* **77**, 1551–1563.
- Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O (1995) A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. *Proc. Natl Acad. Sci. USA* **92**, 9363–9367.
- Dimri GP, Testori A, Acosta M, Campisi J (1996) Replicative senescence, aging and growth-regulatory transcription factors. *Biol. Signals* **5**, 154–162.
- Ferland G, Perea A, Audet M, Tuchweber B (1990) Characterization of liver lysosomal enzyme activity in hepatocytes, Kupffer and endothelial cells during aging: effect of dietary restriction. *Mech. Ageing Dev.* **56**, 143–154.
- Gary RK, Kindell SM (2005) Quantitative assay of senescence-associated β -galactosidase activity in mammalian cell extracts. *Anal. Biochem.* **343**, 329–334.
- Gerland LM, Peyrol S, Lallemand C, Branche R, Magaud JP, Ffrench M (2003) Association of increased autophagic inclusions labeled for β -galactosidase with fibroblastic aging. *Exp. Gerontol.* **38**, 887–895.
- Goodwin EC, Yang E, Lee CJ, Lee HW, DiMaio D, Hwang ES (2000) Rapid induction of senescence in human cervical carcinoma cells. *Proc. Natl Acad. Sci. USA* **97**, 10978–10983.
- Hayflick L, Moorhead PS (1961) The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25**, 585–621.
- Hwang ES (2002) Replicative senescence and senescence-like state induced in cancer-derived cells. *Mech Ageing Dev.* **123**, 1681–1694.
- Hwang ES, Riese DJ, 2nd, Settleman J, Nilson LA, Honig J, Flynn S, DiMaio D (1993) Inhibition of cervical carcinoma cell line proliferation by introduction of a bovine papillomavirus regulatory gene. *J. Virol.* **67**, 3720–3729.

- Kang HT, Lee CJ, Seo EJ, Bahn YJ, Kim HJ, Hwang ES (2004) Transition to an irreversible state of senescence in HeLa cells arrested by repression of HPV E6 and E7 genes. *Mech. Ageing Dev.* **125**, 31–40.
- Knook DL, Sleyster EC, van Noord MJ (1975) Changes in lysosomes during ageing of parenchymal and nonparenchymal liver cells. *Adv. Exp. Med. Biol.* **53**, 155–169.
- Krishna DR, Sperker B, Fritz P, Klotz U (1999) Does pH 6 β -galactosidase activity indicate cell senescence? *Mech. Ageing Dev.* **109**, 113–123.
- Kurz DJ, Decary S, Hong Y, Erusalimski JD (2000) Senescence-associated β -galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. *J. Cell Sci.* **113**, 3613–3622.
- Melk A, Kittikowit W, Sandhu I, Halloran KM, Grimm P, Schmidt BM, Halloran PF (2003) Cell senescence in rat kidneys *in vivo* increases with growth and age despite lack of telomere shortening. *Kidney Int.* **63**, 2134–2143.
- Michaloglou C, Vredeveld LC, Soengas MS, Denoyelle C, Kuilman T, van der Horst CM, Majoor DM, Shay JW, Mooi WJ, Peeper DS (2005) BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature* **436**, 720–724.
- Mishima K, Handa JT, Aotaki-Keen A, Luty GA, Morse LS, Hjelmeland LM (1999) Senescence-associated β -galactosidase histochemistry for the primate eye. *Invest. Ophthalmol. Vis. Sci.* **40**, 1590–1593.
- Norden AG, O'Brien JS (1975) An electrophoretic variant of β -galactosidase with altered catalytic properties in a patient with GM1 gangliosidosis. *Proc. Natl Acad. Sci. USA* **72**, 240–244.
- Oshima A, Yoshida K, Itoh K, Kase R, Sakuraba H, Suzuki Y (1994) Intracellular processing and maturation of mutant gene products in hereditary β galactosidase deficiency (β -galactosidosis). *Hum. Genet.* **93**, 109–114.
- Pendergrass WR, Lane MA, Bodkin NL, Hansen BC, Ingram DK, Roth GS, Yi L, Bin H, Wolf NS (1999) Cellular proliferation potential during aging and caloric restriction in rhesus monkeys (*Macaca mulatta*). *J. Cell Physiol.* **180**, 123–130.
- Robbins E, Levine EM, Eagle H (1970) Morphologic changes accompanying senescence of cultured human diploid cells. *J. Exp. Med.* **131**, 1211–1222.
- Sanchez-Martin MM, Cabezas JA (1997) Evaluation of the activities of eight lysosomal hydrolases in sera of humans, rats and pigs of different ages. *Mech. Ageing Dev.* **15**, 95–107.
- Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW (1997) Oncogenic *ras* provokes premature cell senescence associated with accumulation of p53 and p16^{INK4a}. *Cell* **88**, 593–602.
- Severino J, Allen RG, Balin S, Balin A, Cristofalo VJ (2000) Is β -galactosidase staining a marker of senescence *in vitro* and *in vivo*? *Exp. Cell Res.* **257**, 162–171.
- Shay JW, Roninson IB (2004) Hallmarks of senescence in carcinogenesis and cancer therapy. *Oncogene* **23**, 299–2933.
- Sigal SH, Rajvanshi P, Gorla GR, Sokhi RP, Saxena R, Gebhard DR Jr, Reid LM, Gupta S (1999) Partial hepatectomy-induced polyploidy attenuates hepatocyte replication and activates cell aging events. *Am. J. Physiol.* **276**, G1260–G1272.
- Silva CM, Severini MH, Sopelsa A, Coelho JC, Zaha A, d'Azzo A, Giugliani R (1993) Six novel β -galactosidase gene mutations in Brazilian patients with GM1-gangliosidosis. *Hum. Mutat.* **13**, 401–409.
- Song YS, Lee BY, Hwang ES (2005) Distinct ROS and biochemical profiles in cells undergoing DNA damage-induced senescence and apoptosis. *Mech. Ageing Dev.* **126**, 580–590.
- Turk B, Milo GE (1974) An *in vitro* study of senescent events of human embryonic lung (WI-38) cells. *Arch. Biochem. Biophys.* **161**, 46–53.
- Untergasser G, Gander R, Rumpold H, Heinrich E, Plas E, Berger P (2003) TGF- β cytokines increase senescence-associated β -galactosidase activity in human prostate basal cells by supporting differentiation processes, but not cellular senescence. *Exp. Gerontol.* **38**, 1179–1188.
- Wells SI, Francis DA, Karpov AY, Dowhanick JJ, Benson JD, Howley PM (2000) Papillomavirus E2 induces senescence in HPV-positive cells via pRB- and p21^{CIP}-dependent pathways. *EMBO J.* **19**, 5762–5771.
- Yang N-C, Hu M-L (2005) The limitations and validities of senescence associated β -galactosidase activity as an aging marker for human foreskin fibroblast Hs68 cells. *Exp. Gerontol.* **40**, 813–819.
- Yegorov YE, Akimov SS, Hass R, Zelenin AV, Prudovsky IA (1998) Endogenous β -galactosidase activity in continuously nonproliferating cells. *Exp. Cell Res.* **243**, 207–211.
- Zhang S, McCarter JD, Okamura-Oho Y, Yaghi F, Hinek A, Withers SG, Callahan JW (1994) Kinetic mechanism and characterization of human β -galactosidase precursor secreted by permanently transfected Chinese hamster ovary cells. *Biochem. J.* **304**, 281–288.
- Zhu J, Woods D, McMahon M, Bishop JM (1998) Senescence of human fibroblasts induced by oncogenic Raf. *Genes Dev.* **12**, 2997–3007.
- von Zglinicki T, Nilsson E, Docke WD, Brunk UT (1995) Lipofuscin accumulation and ageing of fibroblasts. *Gerontology* **41**, 95–108.